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- 1) Tam et al, 1989, Proc Natl Acad Sci, USA, 86: 9084-9088.
- 2) Butz et al, Pep Res, 7(1): 20-23, year?

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Vaccine Engineering: Enhancement of Immunogenicity of Synthetic Peptide Vaccines Related to Hepatitis in Chemically Defined Models Consisting of T- and B-Cell Epitopes



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Vaccine engineering: Enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes

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ABSTRACT We report the development of two models for synthetic hepatitis B vaccines. The models were based on the multiple antigen peptide (MAP) system and contained the relevant B- and T-cell epitopes without any macromolecular carrier. Two peptides, representing the a determinant of the S region (S protein) of hepatitis B surface antigen, a dominant serotype of hepatitis B virus infection found in humans, and residues 12-26 of the pre-S(2) region of the middle protein were incorporated as either monoepitope or diepitope MAP models. Immunizations of outbred rabbits with the monoepitope MAP that contains the pre-S(2) antigen resulted in high-titered antibody response to the middle protein, but the other monoepitope, containing only the a-determinant peptide antigen, resulted in poor immune responses to either the peptide antigens or to the S protein. The diepitope MAPs containing both the a and the pre-S(2) determinants produced high-titer antibodies reactive to the a-synthetic peptide and the S protein, as well as to the middle proteins. Thus, our results show that the diepitope MAP models eliminate the need for a protein carrier and that the pre-S(2) peptide determinant serves as a T-helper cell epitope that enhances the immune response of the S region and overcomes the poor immunogenicity encountered with a single epitope of the S region.

A long-term goal for the development of synthetic vaccines is the design of chemically unambiguous, multivalent vaccines containing the relevant B- and T-cell epitopes but requiring no macromolecular carrier. Such synthetic vaccines would eliminate many irrelevant determinants of bacterial and viral vaccines that may cause undesirable side effects. Hepatitis B virus (HBV) represents an important target for a synthetic peptide vaccine model because it affects nearly 200 million people worldwide, and chronic infection produces a risk of developing hepatocellular carcinoma (1). The development of a conventional vaccine has been hindered by the difficulty of growing HBV in tissue culture. Currently available vaccines for HBV include subviral components of inactivated hepatitis B surface antigen (HBsAg) purified from plasma of asymptomatic HBV-infected carriers and surface proteins obtained from the DNA recombinant method. The limitations of both vaccines include production costs and safety in the former case and the incomplete inclusion of all epitopes (e.g., pre-S region) in the latter case (2). A synthetic vaccine based on synthetic peptides may provide an improved design, the necessary supply, and the safety needed for an efficacious vaccine.

The DNA of HBV has a large open reading frame encompassing 1167-1200 base pairs (3, 4). The S gene encodes for the S protein, the major antigen of the HBsAg, which consists of 226 amino acid residues. Two minor but larger envelope proteins (the middle proteins) contain, in addition to the

entire amino acid sequence of the S protein, an NH₂-terminal fragment, the 55-residue pre-S(2), and a 108- to 119-residue pre-S(1), the length of which depends on the HBV strain. The pre-S(1) region is located upstream from the pre-S(2) region, irrespective of subtypes. The virus-neutralizing antibodies are elicited by the S region of the HBsAg as well as the pre-(S) region of the HBV (2-4).

Serologically, HBsAg has one group-specific determinant (a) and two sets of mutually exclusive determinants (d or y and w or r), giving four major serotypes (adw, ayw, adr, and ayr). Bhatnager *et al.* (5) and others (6, 7) reported that residues 139-147 of the S protein contained an essential part of the a determinant of HBsAg. Because most anti-HB response in humans is generally directed against the common determinant a, encountered in all serotypes of HBV, immunization with one serotype of HBsAg is protective against all serotypes. Itoh *et al.* (8) and others (9, 10) reported that a peptide of the pre-S(2) protein (residues 14-32) raised protective antibodies in chimpanzees. These synthetic peptides were coupled to protein carriers such as keyhole limpet hemocyanin for immunization. Because protein carriers are undesirable for human vaccines, we have recently designed a chemically defined approach known as multiple antigen peptide (MAP) system to incorporate peptide antigens that give a macromolecular structure without a large protein carrier (11, 12). MAPs were sufficiently immunogenic and elicit specific monoclonal and polyclonal antibodies to recognize their cognate proteins. MAPs were also designed to allow flexibility to incorporate multiple epitopes in a chemically unambiguous system. In this study, we describe the design of two chemically defined models that incorporate two epitopes of HBsAg, the a determinant of the S region consisting of residues 140-146, and the pre-S(2) antigen consisting of residues 12-26. Our results show that immunization of outbred rabbits with the monoepitope models containing only the a determinant peptide antigen produced no to poor immune responses to either the peptide antigen or to the HBsAg containing the S region, whereas the diepitope models containing both the a and the pre-S(2) determinants produce high-titered antibodies reactive to the synthetic a peptide and the HBsAg, as well as to the pre-S(2) peptide antigen.

MATERIALS AND METHODS

Synthesis of MAP Containing One Epitope. The synthesis of MAP was accomplished manually by a stepwise solid-phase procedure (11-13) on *t*-butoxycarbonyl (Boc)-Ala-OCH₂-Pam resin (14) (0.1 mmol of alanine is present in 1 g of resin).

Abbreviations: MAP, multiple antigen peptide; HBV, hepatitis B virus; HBsAg, hepatitis virus surface antigen; Boc, *t*-butoxycarbonyl; Boc-Lys(Boc), *N*^α,*N*^ε-Boc-Lys; Fmoc, 9-fluorenylmethoxycarbonyl; AcM, acetamidomethyl; DCC, dicyclohexylcarbodiimide.

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After removal of the Boc group by 50% trifluoroacetic acid and neutralization of the resulting salt by diisopropylethylamine (DIEA) the synthesis of the first level of the carrier core to form [Boc-Lys(Boc)]-Ala-OCH₂-Pam resin was achieved using a 4-mol excess of Boc-Lys(Boc) via dicyclohexylcarbodiimide (DCC) alone in CH₂Cl₂. The second and third levels were synthesized by the same protocol to give the octabranching Boc-Lys(Boc) core matrix. For synthesizing the core matrix of a MAP containing two different antigen sequences, 9-fluorenylmethoxycarbonyl (Fmoc)-Lys(Boc) was used in the third level to give Fmoc-Lys(Boc) end groups. The protecting group scheme for synthesis of antigen was as follows: Boc group for the α -NH₂ terminus and side-chain protecting groups for the trifunctional amino acids were: Glu(benzyl ester), Lys(*p*-chlorobenzoyloxycarbonyl), Thr(benzyl), Asp(benzyl ester), Cys(acetamidomethyl; Ac_m), Arg(tosyl), and Tyr(*p*-bromobenzoyloxycarbonyl). Deprotection by trifluoroacetic acid was preceded by one trifluoroacetic acid prewash. Neutralization by DIEA was in dimethylformamide. The coupling was mediated with DCC/1-hydroxybenzotriazole in dimethylformamide. After completion of the synthesis, the MAP-resin was treated with trifluoroacetic acid to remove the N^α-Boc groups, then acetylated with 10% acetic anhydride/10% DIEA in CH₂Cl₂, and finally cleaved with the low-high HF method (15) to remove the MAP from the resin support. The crude peptide was then washed with cold ether/mercaptoethanol (99:1 vol/vol) to remove *p*-thiocresol and *p*-cresol and extracted into 8 M urea in 0.1 M Tris-HCl buffer (pH 8.0). To remove the remaining aromatic by-products generated in the cleavage step, MAPs were dialyzed (Spectra Por 6, *M*, cutoff 1000) in 8 M urea and then in 0.1 M acetic acid twice for 5–6 hr to remove the urea. The MAPs were lyophilized in H₂O three times to remove acetic acid.

Synthesis of MAP Containing Two Different Epitopes. For synthesis of the core matrix of a MAP containing two different epitopes, Fmoc-Lys(Boc) was used to selectively protect the lysine branching of the core at the third level to give four Lys(Boc) and four Fmoc-Lys end groups. The synthesis of the first epitope used the Boc chemistry as just described, but during this synthesis, neutralization time was reduced to 1 min to minimize the premature cleavage of the Fmoc group. The synthesis of the second epitope used the Fmoc chemistry (16) and started after the completion of the Boc-amino acid chain was assembled. The Fmoc-amino acids were used with the side-chain protecting groups for the

trifunctional amino acids as follows: Glu(*tert*-butyl ester), Asp(*tert*-butyl ester), Lys(Boc), Thr(*tert*-butyl ester). Deprotection by piperidine was preceded by one piperidine prewash, and the coupling was mediated with DCC/1-hydroxybenzotriazole in dimethylformamide. After completion of synthesis, the MAP-resin was treated with low-high HF (15) to remove the peptide chains from the resin. The procedure for Fmoc amino acid peptide chain was as follows: (i) 20 ml of dimethylformamide (three times for 1 min); (ii) 20 ml of piperidine/dimethylformamide (1:1, vol/vol) (1 min); (iii) 20 ml of piperidine/dimethylformamide (1:1, vol/vol) (10 min); (iv) 20 ml of dimethylformamide (three times for 1 min); (v) 20 ml of CH₂Cl₂ (three times for 1 min); (vi) 20 ml of dimethylformamide (two times for 1 min); (vii) amino acid (4 equivalents) of 5 ml of dimethylformamide (5 min), 1-hydroxybenzotriazole (4 equivalents) in dimethylformamide, DCC (4 equivalents) in CH₂Cl₂ were added for 2 hr; (viii) 20 ml of dimethylformamide (four times for 2 min); (ix) 20 ml of CH₂Cl₂ (two times for 2 min).

Dimerization of Two Heterologous Epitope MAPs by Oxidation to the Disulfide. To 1 μ mol of MAP, the diepitope containing Cys(Ac_m) dissolved in a deaerated and N₂-purified 50% acetic acid solution at room temperature, 500 μ l of a solution of I₂ in MeOH (1 mM solution) was added batchwise for 1 hr at 0°C. The reaction was quenched by adding 1 M aqueous sodium thiosulfate until the yellow color was removed. MeOH was removed by dialysis in 0.1 M acetic acid, and the desired MAPs were purified by gel-permeation chromatography. All MAPs gave a satisfactory amino acid analysis.

Immunization and Assays. Rabbits (New Zealand White, two for each antigen) were immunized by s.c. injection (0.5 ml) of the MAP (0.5 mg in 1 ml of phosphate-buffered saline) in complete Freund's adjuvant (1:1) on day 0 and in incomplete Freund's adjuvant (1:1) on days 21 and 42 and were bled on day 49. The antisera were used without purification. An enzyme-linked immunoabsorbent assay (ELISA) was used to test antisera for ability to react with the MAP (0.5 μ g per well), linear peptide, recombinant major, and middle antigens (gifts from Merck Sharp & Dohme).

RESULTS

Model Design Incorporating Two Epitopes. Two models (Fig. 1 and 2) referred to as homologous and heterologous branching epitopes were designed to produce MAPs contain-

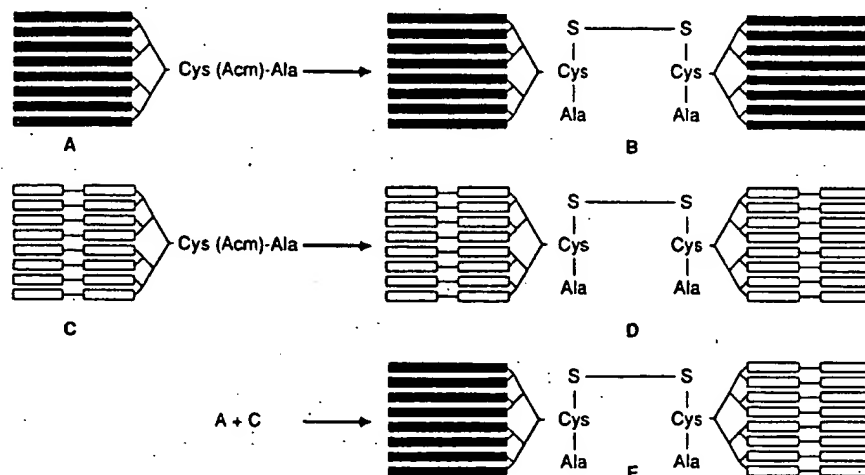


Fig. 1. Structure of MAPs from the homologous branching approach. The two peptides being used are TN14 (TKPTDGN)₂ (a tandem repeating sequence of residues 140–146 of the S region in one-letter code) and LG15 (LQDPRVRGLYFPAGG) (residues 12–26 of pre-S(2) region).

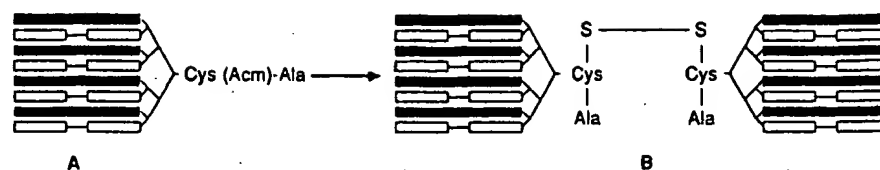


FIG. 2. Structure of MAPs from the heterologous branching approach.

ing two different peptide epitopes. For these models we used two different epitopes of HBV envelope proteins, a 14-residue peptide representing a tandemly repeating 7-residue peptide of residues 140–146 of the a determinant of the S region (designated as TN14), and a 15-residue peptide of the pre-S(2) protein covering residues 12–26 (designated as LG15). The antigen LG15 or TN14 was synthesized as an acetylated form in an octabranching MAP format (Fig. 1 A and C). LG15 represents a continuous segment of the pre-S(2) sequence, and TN14 represents two tandemly repeating segments of residues 140–146 of the S region (two cysteines normally occurring at positions 139 and 147 were avoided to simplify our synthesis). The design of tandemly repeating segments of TN14 would compensate for the disparity in length between LG15 (a 15-residue peptide) and the sequence of 140–146 of the S region (a 7-residue peptide).

Homologous branching. In the homologous branching model (Fig. 1), one antigen was branched homologously to give eight copies of dendritic forms of antigens attached to the heptalysyl core such as the acetylated LG15-MAP (Fig. 1A) or TN14-MAP (Fig. 1C). Each homologous branched MAP contained a single copy of a dipeptide, Cys(Acm)-Ala, at its COOH terminus. The removal of the S-Acm protecting group and concomitant oxidation by I_2 produces a homomeric dimer of the MAP (Fig. 1 B and D). However, when two different homologous branched MAPs, LG15-MAP and TN14-MAP, are mixed in an equal molar ratio and subjected to the oxidative disulfide formation by I_2 , it produces mainly a heterodimer containing a diepitope MAP consisting of LG15-MAP and TN14-MAP, such as LG15/TN14-MAP (Fig. 1E). The oxidation reaction was not quantitative and required purification of the desired heterodimer from the homodimers by gel-permeation chromatography.

Heterologous branching. In the heterologous branching model (Fig. 2), four copies of two different antigens, LG15

and TN14, were attached to the same octabranching MAP to give a diepitope MAP with a design of alternating antigens (Fig. 2A). For comparison, we have also prepared the dimer (Fig. 2B). The key was to use an orthogonal protecting group approach in the synthesis for each antigen so that when the synthesis of one antigen was completed, the synthesis of the other antigen could be achieved without affecting the integrity of the first antigen. This was achieved by using two sets of protecting group combinations, Fmoc/*tert*-butyl ester and Boc/benzyl.

The synthesis of the peptide antigens began with the heptalysine core already attached to a resin such as the Pam resin. However, there was a deviation from the usual synthesis of the MAP core, which was uniformly protected at the NH_2 terminals with the Boc group. The protecting groups of the N^α - and N^ϵ -amino groups in this case was orthogonally protected with Boc and Fmoc groups, respectively. Under these circumstances, the Boc group was cleaved in acid, and the peptide chains were elongated at the N^α -amino groups without affecting the N^ϵ -amino groups. Once the assemblage of the first peptide antigen chains were completed, the Fmoc groups were then cleaved with piperidine to begin the synthesis of the second peptide epitope. After the complete synthesis of the second peptide antigen chains, the amino groups were capped and the MAP containing the peptide antigens was cleaved off from the resin, purified, and used as immunogen.

Enhanced Antibody Response of the Diepitope MAP Models. Antibodies from New Zealand White rabbits immunized with each of the seven MAP models emulsified with complete Freund's adjuvant were tested after 7 weeks for titers reactive with their respective MAP immunogens, the monomeric synthetic peptides LG15 or TN14, or with recombinant S and middle proteins. The inclusion of the synthetic peptide monomers as well as the native HBsAgs provided the required

Table 1. Immunological responses of different antisera

MAP model	Serum titer* $\times 10^{-3}$					
	Monomeric peptide		MAP		Native protein	
	LG15	TN14	LG15	TN14	S protein	M protein
Single epitope						
LG15(8)-MAP (1A)	5	<0.2	65	<0.2	<0.3	34
LG15(8)-MAP-2 (1B)	8	<0.2	51	<0.2	<0.2	72
TN14(8)-MAP (1C)	<0.3	<0.3	<0.2	<0.2	<0.2	<0.2
TN14(8)-MAP-2 (1D)	<0.3	<0.2	<0.3	<0.2	<0.2	<0.2
Double epitopes						
LG15(8)-MAP-TN14(8) (1E)	17	2.6	23	41	5.2	45
LG15(4)/TN14(4)-MAP (2A)	4.1	5.5	16	18	3.2	64
LG15(4)/TN14(4)-MAP-2 (2B)	5.6	6.4	25	7.2	6.4	13
Mixed epitopes (noncovalent)						
1A + 1C	3	<0.2	42	<0.2	<0.2	24
1B + 1D	11	<0.2	37	<0.2	<0.2	15

The system used to name different MAP models can be generalized as follows: antigens (no. of branches)-MAP-monomer or dimer. For convenience, dimer is indicated by the numeral 2. Thus, model 1A or 1C contains eight homomeric branching antigens of either LG15 or TN14, and model 1B or 1D is the respective dimer of 1A and 1C. Similarly, model 2A contains four branches each of antigen LG15 and TN14, and model 2B is its dimer. Model 1E is the heterodimer of model 1A and 1C linked by a disulfide bond (see Figs. 1 or 2 for the schematic representation of each model).

*Titers were expressed as the reciprocal of the highest serum dilution.

criteria for the specificity of the antibody response to the sequence and the protein from which the antigens are derived.

The antibody response could be classified into three groups (Table 1). The first group, monoepitope LG15-MAP, elicited strong antibody response with titers at about $1:10^4$ dilution to LG15-MAP, the synthetic peptide LG15, and the middle protein consisting of the pre-S(2) and S region but not to TN14-MAP or S protein. The second group, monoepitope TN14-MAPs, elicited no response to TN14-MAPs, the monomeric synthetic peptide TN14, or the S protein consisting only of the S region or to the LG15 antigens. The third group, the diepitope MAPs, consisting of covalently linked epitopes, elicited the strongest responses with titers at ≈ 1 to 10^5 dilution, to synthetic peptides TN14 and LG15 and both S and middle proteins. Thus, the diepitope MAPs overcame the poor immunogenicity of TN14 and elicited antibody responses to both antigens. The nonresponsiveness of the animals immunized with the TN14-MAPs could not be overcome with a noncovalent mixture consisting of various combinations of monoepitope MAPs of TN14 and LG15.

The secondary antibody response (Table 1) after boosting with MAPs in incomplete Freund's adjuvant did not result in any change in the pattern seen in the primary responses. The monoepitope MAPs of TN14 produced no significant antibody response, whereas the diepitope MAPs continued to elicit antibody responses to both determinants at ≈ 5 - to 10-fold higher than those of the primary titers. Similar results were obtained from antibodies obtained after the second and third boosts at 3-week intervals.

Specificity of Antibodies of the Diepitope MAP Models to HBsAg. To further show the specificity of the antibodies obtained from the diepitope MAPs, we used various concentrations of these antibodies to immunoblot the HBsAg in SDS/PAGE. As shown in Fig. 3, the antisera of the diepitope MAPs recognized the HBsAg containing the S region or HBsAg containing both pre-S(2) and S region. Preimmune antiserum or the anti-LG15-MAP antiserum did not result in any reaction. However, anti-TN14-MAP antisera weakly recognized the denatured major and middle proteins. Preincubation of the diepitope MAP antisera with the corresponding synthetic peptide TN14 or LG15 abolished the recognition.

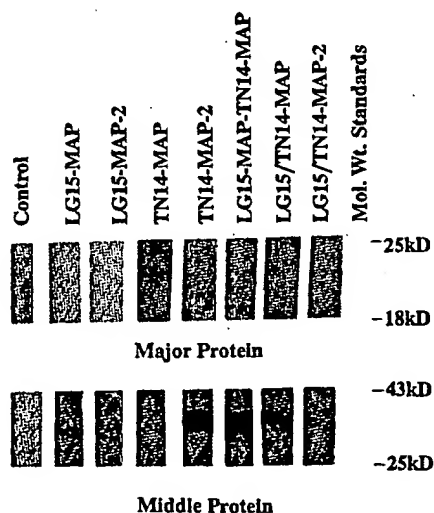


Fig. 3. Immunoblotting of recombinant S and middle proteins with different antisera from MAPs. Preincubation of MAP antisera with the corresponding synthetic peptide TN14 or LG15 abolished recognition.

DISCUSSION

The results described in this paper using the MAP system provide a general approach to the design of synthetic vaccines. (i) This approach allows the incorporation of multiple copies of two or more epitopes in a defined and deliberate manner. Furthermore, this approach also allows the flexibility of interchanging epitopes by selectively dimerizing two types of epitopes. Such an approach would be particularly useful for testing different B epitopes with one universal T epitope, or vice versa. (ii) Clearly the arrangements for incorporation of multiple antigens into the MAP system are numerous, but the basic chemistry involved will be variations of the combination of Boc/benzyl and Fmoc/*tert*-butyl strategies. (iii) The resulting heteromeric MAP, containing two or more peptide antigens, may elicit in animals very different immunological responses from the homomeric MAP containing only one peptide antigen.

The design of a peptide-based synthetic vaccine requires the inclusion of relevant immunogenic epitopes. In our study we selected two hepatitis B epitopes, the a determinant from the S region and residues 12-26 of the pre-S(2) region. The results show the flexibility and versatility of the MAP approach to incorporate these epitopes in a chemically defined manner. Furthermore, we also show that the diepitope arrangements enhanced immune responses of the a determinant.

The a determinant, contained in the S region, which is relatively cysteine-rich, is disulfide-dependent. Disruption of the disulfide restraints greatly affects its antigenicity (1, 5-7). The pre-S(2) determinant in the pre-S(2) region, which does not contain any cysteine residue, is disulfide independent. Consequently, its antigenicity is more likely to be reproducible by synthetic peptide antigens (8-10). The immunogenicity of and protective efficacy of antibodies elicited by the pre-S(2) region have been taken into consideration in the design of the diepitope MAP models. Indeed, peptide antigens from the pre-S region have been shown to be highly immunogenic and offer protection to HBV in the chimpanzee models (9, 10). Furthermore, the pre-S region is highly conserved, and its peptide antigen prevents infection against all strains of the four subtypes efficiently. Moreover, the pre-S region is absent from the presently produced subunit recombinant vaccine, and this provides a strong basis for its inclusion in the synthetic peptide vaccine.

The universally low response in animals, including rabbits, immunized with synthetic peptide antigens of the a or other regions of the S gene product of HBV when such peptide antigens are conjugated to a protein carrier has been reported by several groups (5-7). The results of our present study using the monoepitope MAP system confirm that a linear peptide representing the a determinant is a poor immunogen and produces little antibody response either against the peptide antigen or the cognate protein. However, diepitope MAPs consisting of a peptide of the a determinant and a peptide of pre-S(2) region can stimulate high antibody response to the S region. Thus, our results indicate that the pre-S(2) region of the HBsAg overcame the nonresponsiveness of B-cell epitopes of the S region and may serve as a T-helper cell epitope. Enhanced immunogenicity of the S region by preimmunization with pre-S protein has been shown by Milich *et al.* (17, 18) in inbred strains of mice. They show that preimmunization with pre-S protein of mice nonresponsive to S protein results in an enhanced response to S protein after reimmunization with HBsAg containing both proteins. Furthermore, enhanced immunization by the pre-S(2) region at the T-cell level as a T-helper cell epitope was observed in inbred mice strains using a recombinant protein containing the whole pre-S(2) and S regions (17). The poten-

tiating effect of pre-S(2) has not been seen in outbred rabbit species.

The design of a diepitope MAP system containing a T-cell epitope such as the pre-S(2) sequence and a B-cell epitope such as the a determinant circumvented the nonresponsiveness of the B epitope by providing the T-helper function (19, 20). It also appears that the pre-S(2) epitope also serves as a carrier for the a determinant. One function of protein carriers, such as serum albumin, keyhole limpet hemocyanin, or tetanus toxoid, is often to enhance the immunogenicity of the synthetic peptides (21, 22) by providing a T-helper cell epitope. However, such carriers are not suitable for use in human vaccines. The inclusion of a protein carrier, besides the disadvantage of chemical ambiguity, can lead to the complications of hypersensitivity to the carrier and epitopic suppression. The diepitope MAPs would generally overcome these disadvantages and provide defined models for the design and engineering of synthetic vaccines.

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